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GROWTH MEDIA DEPENDENT CHARACTERISTICS OF *NEISSERIA MENINGITIDIS* (U)

by

M.H. Knodel, J. Fildes, M.R. Spence, L.A. White and A.R. Bhatti

ACN No. 16A10

December 1983

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ABSTRACT

Four disease isolates of *Neisseria meningitidis* were characterized on the basis of their virulence in mice and sensitivity to antibiotics. Repeated subculturing of a virulent strain resulted in the loss of pili and extracellular material with the subsequent loss of virulence properties of the organism. Extracellular material associated with the virulent strain seems to play an important role in infection in mice. Strains resistant to sulfonamides were also found to be resistant to combination of sulfonamides with trimethoprim. This is a new finding. *N. meningitidis* showed an increased sensitivity to antibiotics on a chemically defined medium as compared to an enrichment medium. The changes were dependent on the nature of the growth medium and were reversible. This increased sensitivity of *N. meningitidis* to antibiotics on chemically defined media may be due to permeability changes occurring in the outer membranes of the organism.

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INTRODUCTION

Neisseria meningitidis is a non-motile, non-sporeforming, gram negative diplococcus which is oxidase and catalase positive, produces acid but no gas from glucose and maltose and no acid from sucrose or fructose (Catlin, 1973). Clinical isolates of *N. meningitidis* display a great deal of antigenic diversity based on their surface structures and chemical differences in the outer cell wall proteins, capsule, lipopolysaccharide, and pili. The species has been divided into ten different serogroups based on the immunological specificity of the capsular polysaccharide antigens (Branham, 1953; Catlin, 1974; Morello and Bohnhoff, 1980). *N. meningitidis* can colonize the nasopharyngeal area without a pathogenic outcome, resulting in the formation of a carrier state and/or allows for the development of host immunity. The carrier state is the major reservoir of this organism and transmission of meningococci is usually facilitated

by aerosol or contact with respiratory secretions (Morello and Bohnhoff, 1980). In this communication, we describe the virulence of serogroup A, *N. meningitidis*, for mice and antibiotic sensitivity with respect to the nature of the growth media for some disease isolates.

MATERIALS AND METHODS

Bacterial Strains: *N. meningitidis* strains used in this study and their sources are as follows: strains SP-3428, piliated and non-piliated variants which were segregants from SP-3428, a disease case isolate from Sao Paulo, Brazil and were obtained from Dr. I.W. DeVoe, McGill University, Montreal (DeVoe and Gilchrist, 1978). Strain LCDC-604, a disease isolate, was obtained from the Laboratory Center for Disease Control (LCDC), Ottawa, Canada. Strain DRES-30, another disease isolate, was isolated at the Defence Research Establishment Suffield (DRES) from a blood sample from a patient suffering from a suspected laboratory acquired infection. Stock cultures were prepared by lyophilizing strains after 1 to 3 initial subcultures and storing these at 4°C.

Preparation of Cultures for Infectious Study: Cultures were grown on Columbia Blood Agar supplemented with 4% (v/v) sheep erythrocytes and 1% Iso-Vitalex (CBA media) in tissue culture flasks as described previously (Holbein, Jericho and Likes, 1979). Bacterial cells were dislodged from the agar surface by gentle rocking in 10 mL of Neisseria chemically defined medium (NCDM) containing about 25 sterile glass beads. The harvested cell suspension contained approximately 10^{10} colony-forming units (CFU) of *N. meningitidis* per mL. Dilutions were prepared in NCDM and viable counts as CFU were determined after over-night growth on CBA at 35°C in 5% CO_2-95% air.

The infectivity of different strains of *N. meningitidis* was studied in groups of 5 to 10 mice, each weighing 17 to 20 gms, as described by Holbein *et al.*, 1979. Each group of mice was injected with 0.5 mL of iron dextran (approximately 250 mg of iron per kg mouse weight), immediately followed by intraperitoneal injection of 0.5 mL of bacterial suspension. Infecting doses ranged from 1 to 10^9 CFU per mouse. Controls received iron dextran only. The general physiological condition of the mice was observed and the mortality scored at 24, 48 and 72 h after infection. LD_{50}'s, in terms of CFU, were determined by probit analysis (Holbein *et al.*, 1979).

Antibiotic Sensitivity: The sensitivity of different strains of *N. meningitidis* to a variety of antibiotics was determined on CBA medium according to the system regulated by the National Committee for Clinical Laboratory Standards (1979) and based on the paper disc technique described by Bauer *et al.*, 1966.

Electron Microscopy: Formalin-killed *N. meningitidis* suspensions were negatively stained on Formvar grids stabilized with carbon using 2% sodium phosphotungstate (w/v), pH 7.2. Specimens were examined in a JOEL JEM-100CX electron microscope operating at 60 kV.

Chemicals and Reagents: Iso-Vitalex was purchased from Baltimore Biological Laboratory, Cockeysville, Md. Columbia blood agar and NCDM were obtained from Grand Island Biological Co., Grand Island, N.Y. Iron dextran was purchased from Dextran Products Ltd., Scarborough, Canada. Antibiotic sensitivity discs were purchased from Becton, Dickinson and Co. Canada Ltd., Mississauga, Ontario, Canada.

RESULTS AND DISCUSSION

Electron microscopic examination of negatively stained cultures revealed that over 85% of the DRES-30 cells were piliated, whereas strains LCDC-604, SP-3428 (non-piliated) SP-3428 (piliated) gave 60 – 75%, 50 – 60% and over 80% piliated cells, respectively. The numbers of pili per bacterial cell ranged from 1 – 10 in the three latter strains and bundles of 4 – 8 pili were frequently seen. The length of pili also varied. DRES-30, on the other hand, frequently possessed between 10 – 20 pili per cell. The virulence of each strain was determined, and the LD_{50}'s obtained for the various strains are presented in Table 1. Strains DRES-30, SP-3428 (piliated) and LCDC-604 were highly virulent, whereas SP-3428 (non-piliated) showed medium virulence in mice. These results are comparable to those previously reported for disease isolates of *N. meningitidis* (Holbein, 1981). The isolate from the presumed laboratory acquired infection, DRES-30, showed high virulence with an LD_{50} more similar to that of SP-3428 (piliated) and LCDC-604 than SP-3428 (non-piliated).

Iron has been implicated in bacterial infection and in the host-parasite relationship (Holbein, 1980; Payne and Finkelstein, 1978; Weinberg, 1978). Therefore, the effect of iron was investigated for DRES-30 and LCDC-604. It can readily be seen that iron

enhanced the virulence (Payne and Finkelstein, 1978; Holbein *et al.*, 1979) of tested strains (Table 1). In the present study, *N. meningitidis* was found to be piliated on initial culture whether from disease cases or from carriers, a phenomenon which has previously been reported by DeVoe and Gilchrist (1974). As pili have been documented as an important virulence factor in various gram-negative bacteria (Marx, 1980), their role was investigated in DRES-30. *N. meningitidis* DRES-30 was subcultured seven times on CBA medium. After the seventh sub-culture, 96% of the cells of this strain were devoid of pili (Fig. 1B). As reported previously, (McGee *et al.*, 1977), the loss of pili does not result in a change in colony morphology with respect to size and appearance. Piliated cells showed a variety of pilar lengths, and they were found to be surrounded by an extra-cellular material (ECM) (Fig. 1A). This ECM was not seen around the non-piliated cells (Fig. 1B). When these piliated and non-piliated variants of DRES-30 were tested for their virulence in mice, DRES-30 sub 7, a non-piliated variant gave low virulence as compared to parent strain (Table 2). The decreased virulence observed in the present study cannot solely be attributed to the loss of pili since the large amounts of ECM present in the initial culture (Fig. 1A) were found to be absent after the seventh sub-culture (Fig. 1B). The loss of virulence in DRES-30 after subculturing was assumed to be due to either the loss of pili or certain antigens associated with the ECM or both. Although there is a direct correlation between the presence of pili and virulence in *N. gonorrhoeae*, both for humans (Kellogg *et al.*, 1963; Swanson, Kraus and Gotchlich, 1971) and for chick embryos (Buchanan and Gotschlich, 1973), there is no conclusive evidence to date which directly links pili to the ability of *N. gonorrhoeae* to cause infection in the host (Novotny, Short and Walker, 1975). In a previous study, most of the *N. meningitidis* strains isolated from carriers were found to be heavily piliated but avirulent (Holbein *et al.*, 1979) thus indicating that virulence is not directly related to the presence of pili. Therefore, detailed studies on the ECM associated with the virulent strains are warranted to elucidate any possible role which it plays in virulence. However, the role of pili in the attachment to the host tissue as an important initial step in the infection process cannot be ruled out.

Sensitivity or resistance to antibiotics among gram-negative bacteria is frequently a function of permeability (Hare, 1978) which in turn depends on growth conditions (Ingram, Bhatti and DeVoe, 1976) as well as aerosol stress (unpublished results). These factors have been observed to affect the sensitivity of *N. meningitidis* to antibiotics. Results presented in Table 3 show that all four strains tested gave the same general

pattern of inhibition and resistance to various antibiotics. Certain exceptions were noted, however. Strain SP-3428 (non-piliated) was found to be sensitive to gentamicin, whereas the other three strains were resistant to this antibiotic. In contrast to other strains, LCDC-604 was also found to be sensitive to kanamycin, sulfathiazole and the combination of trimethoprim + sulfamethoxazole. Contrary to earlier reports (Souther and Kutscher, 1970; Solberg and Anderson, 1981), the sulfonamide resistant strains of *N. meningitidis*, were found more resistant to other antibiotics than the sulfonamide-sensitive ones. Feldman (1973) has demonstrated that meningococci which were markedly resistant to sulfonamides were inhibited by the combination of sulfonamides with trimethoprim. Such inhibition was not observed with strains SP-3428 piliated, SP-3428 non-piliated and DRES-30 in the present study. Resistance to sulfonamides may be due to the low affinity of sulfonamides for folic acid synthetase, changes in the feed-back or tranport mechanisms, or to overproduction of p-aminobenzoic acid (Davies and Smith, 1978; Garrod, Lambert and O'Grady, 1973). In gram-negative bacteria, however, R-factor resistance may develop rapidly, and plasmid mediated sulfonamide resistance is very common (Widh and Skold, 1977). It is generally accepted that most resistance characters are likely to be transposable. The mechanism of sulfonamide resistance in *N. gonorrhoeae* group is unknown, however, but has never been shown to be associated with a plasmid (Davies and Smith, 1978). Small molecular weight plasmids have recently been demonstrated in *N. meningitidis* (Bhatti, *et al.*, 1981; Verschueren *et al.*, 1982). However, these plasmids have not been studied for antibiotic resistence.

The medium of growth frequently affects the physiology and morphology of microorganisms (Melling and Brown, 1975). Therefore, the antibiotic sensitivity of DRES-30 and LCDC-604 was further compared using CBA and the NDM-agar media of Archibald and DeVoe (1978) (Table 4). *N. meningitidis* grows very slowly on NDM as compared to CBA. On CBA, results are comparable to the results obtained in the previous experiments. As compared to CBA, when both strains were tested on NDM-agar media, they showed sensitivity to a wider range of antibiotics, eg., gentamicin, kanamycin, streptomycin and vancomycin. DRES-30 which was resistant to trimethoprim + sulfamethoxazole on CBA media, became sensitive to these antibiotics when cultured on NDM agar media. However, by comparing the respective zone of inhibitions on the two media, it appears quite evident that DRES-30 is less sensitive to the antibiotics cephaloridine, sulfadiazine and sulfathiazole than is LCDC-609. One way

analysis of variance also sl wed a significant difference (P = 0.001) between the media used. In another set of experiments, when NDM-agar grown cells of these strains were plated on CBA medium and tested for sensitivity to gentamicin, kanamycin, streptomycin and vancomycin, it was found that these strains became resistant to these antibiotics (data comparable to Table 4). Based on these observations, it is suggested that the antibiotic sensitivities observed for the *N. meningitidis* strains are growth medium dependent and reversible.

These antibiotic sensitivity changes could be attributed to permeability changes occurring in the outer cell wall membranes during growth in different media. To elucidate this point, studies are currently underway at both the electron microscopic and biochemical levels.

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TABLE 1

VIRULENCE OF *N. MENINGITIDIS* STRAINS IN MICE

STRAIN	SEROGROUP	IRON	LD_{50} (CFU)	VIRULENCE
DRES-30	A	+	4.3×10^3	High
		−	$\geqslant 10^8$	None
LCDC-604	A	+	3.1×10^3	High
		−	$\geqslant 10^8$	None
SP-3428 (piliated)	A	+	1.5×10^3	High
		ND		
SP-3428 (non-piliated)		+	1.1×10^5	Medium
		ND		

ND, not determined

TABLE 2

EFFECT OF SUB-CULTURE ON VIRULENCE OF *N. MENINGITIDIS* DRES-30

SUB-CULTURE	LD_{50} (CFU)	DIFFERENCES
1	1.9×10^3	Pili and extra-cellular material
7	4.1×10^6	Loss of Pili; Loss of extra-cellular material

TABLE 3

ANTIBIOTIC SENSITIVITY OF *N. MENINGITIDIS*

ANTIBIOTIC		STRAIN			
		SP-3428 (piliated)	SP-3428 (non-piliated)	DRES-30	LCDC-604
Gentamicin	(10 μg)	—	9	—	—
Kanamycin	(30 μg)	—	—	—	8
Sulfathiazole	(300 μg)	—	—	—	10
Trimethoprim + Sulfamethoxazole	(1.25 μg) (23.75 μg)	—	—	—	16
Penicillin G	(10 μg)	20	20	20	28
Ampicillin	(10 μg)	19	20	20	26
Rifampin	(5 μg)	18	18	20	20

— These strains of *N. meningitidis* were found to be resistent to colymycin, lincomycin, mycostatin and streptomycin.

— Zones of inhibition are given in mm and were estimated after 24 h of incubation at 37°C.

TABLE 4

EFFECT OF GROWTH MEDIUM ON THE ANTIBIOTIC SENSITIVITY OF *N. MENINGITIDIS*

ANTIBIOTICS		STRAINS			
		DRES-30		LCDC-604	
		CBA	NDM	CBA	NDM
Ampicillin	10 μg	20	25	26	30
Bacitracin	5 U	8	12	8	9
Cephaloridin	30 μg	8	15	8	30
Chloromycetin	10 μg	26	30	30	30
Gentamycin	10 μg	—	9	—	8
Kanamycin	30 μg	—	10	—	9
Penicillin G	10 U	20	30	28	*
Streptomycin	10 μg	—	9	—	8
Sulfadiazine	50 μg	—	—	8	30
Sulfathiazole	30 μg	—	18	10	30
Tetracycline	10 μg	15	25	18	25
Trimethoprim + Sulfamethoxazole	1.25 μg 23.75 μg	—	15	16	20
Rifampin	5 μg	20	18	20	25
Vancomycin	30 μg	—	18	—	8

— Initial cultures were grown on CBA medium. Cells were harvested (as described in Materials and Methods) and plated on CBA and NDM-agar, respectively, and tested for antibiotic sensitivity. Zones of inhibition were recorded after 24 h of incubation at 37°C.

— *N. meningitidis* was resistent to colymycin, lincomycin, mycostatin and streptomycin.

— Zones of inhibition are given in mm and were estimated after 24 h of incubation at 37°C.

* Irregular growth and large zone of inhibition.

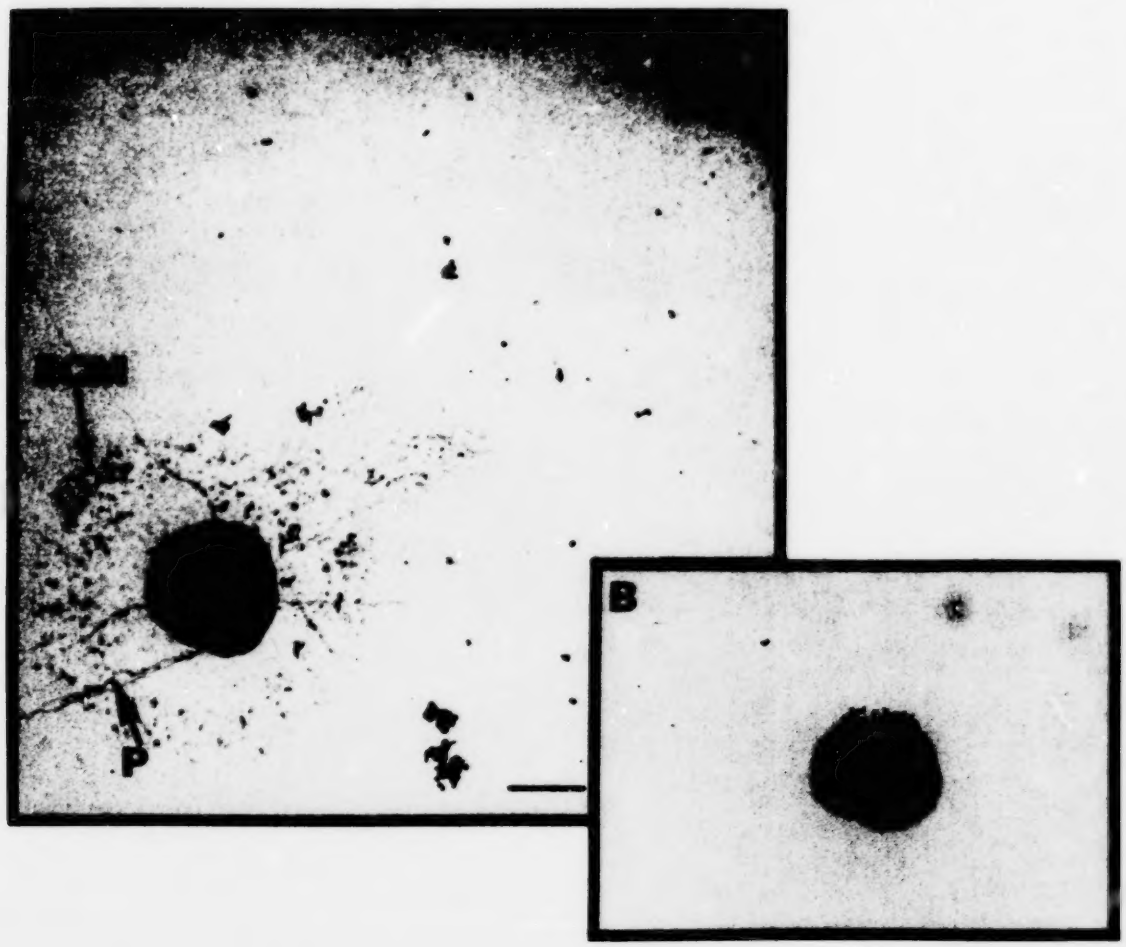

Figure 1

Electron micrograph of negatively stained cell of *N. meningitidis*. DRES-30 with single and bundle of pili "P" and surrounded by extra-cellular material "ECM" (A); and after the 7th subculture (B). The bar represents 0.5 μm.

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1 ORIGINATING ACTIVITY DRES	2a. DOCUMENT SECURITY CLASSIFICATION Unclassified 2b. GROUP

3 DOCUMENT TITLE

Growth Media Dependent Characteristics of Neisseria meningitidis

4 DESCRIPTIVE NOTES (Type of report and inclusive dates)

5 AUTHOR(S) (Last name, first name, middle initial)

M.H. Knodel, J. Fildes, M.R. Spence, L.A. White and A.R. Bhatti

6 DOCUMENT DATE December 1983	7a. TOTAL NO. OF PAGES 18	7b. NO. OF REFS 33
8a. PROJECT OR GRANT NO. 16A10	9a. ORIGINATOR'S DOCUMENT NUMBER(S) SM 1096	
8b CONTRACT NO.	9b. OTHER DOCUMENT NO.(S) (Any other numbers that may be assigned this document)	

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